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Putative Transcriptional Regulator LysR1 Is Required for Full Virulence of *Erwinia amylovora*

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ABSTRACT

Fire blight, caused by the gram-negative bacterium *Erwinia amylovora*, is a serious disease of apple and pear trees. Here, we report genetic evidence that the predicted transcription factor LysR1 is required for full *E. amylovora* virulence. A transposon insertion mutant and an allelic exchange mutant of *lysR1* exhibited reduced virulence compared to the wild type in host apple tissues and a plasmid-borne copy of *lysR1* restored virulence to the mutants. Unexpectedly, the *lysR1* mutants were not auxotrophic for lysine. Numerous virulence factors, such as type III secretion system function, swimming motility and lipopolysaccharide and levansucrase enzyme production, were unchanged in *lysR1* mutants compared to the wild type. However, *lysR1* mutants were specifically deficient in production of the exopolysaccharide amylovoran, an essential *E. amylovora* virulence factor. Global gene expression pattern analysis using RNA sequencing revealed 34 differentially expressed genes between a *lysR1* mutant and the wild type growing in culture. Expression of *lysA*, the presumptive LysR1 regulatory target, was reduced but not abolished in the *lysR1* mutant. No expression differences were detected in genes involved in amylovoran biosynthesis or regulation, and none of the other differentially expressed genes had obvious links to *E. amylovora* virulence. The decreased amylovoran production in *lysR1* mutants is presumably an indirect effect of loss of LysR1 activity, because LysR1 does not appear to be required for the expression of amylovoran biosynthesis genes. Nonetheless, amylovoran deficiency most likely contributes to the virulence defect of *lysR1* mutants and could be the major factor leading to the virulence reduction.

1 | Introduction

Fire blight is an economically impactful disease that affects apple (*Malus domestica*) and pear (*Pyrus communis*) production across many parts of the world. A study published in 2003 estimated the annual costs due to fire blight crop loss and management at about $100 million annually in the United States alone, a number that has undoubtedly increased over the years (Norelli et al. 2003). The disease is caused by the gram-negative bacterium *Erwinia amylovora*, which has historical interest as the first bacterium shown to be the cause of a plant disease (Burrill 1880). Fire blight management tools are limited and

include antibiotic sprays at bloom time to prevent floral infections and removal of infected tissues during winter (reviewed by van der Zwet et al. 2012).

The genetic underpinnings of *E. amylovora* virulence are a matter of ongoing study, with additional genes contributing to virulence continuing to be identified (e.g., Kharadi et al. 2022). During ongoing screening of *E. amylovora* Tn*5* transposon insertion mutants for reduced virulence using an immature apple fruitlet fire blight model system (Klee, Sinn, and McNellis 2019), we identified a mutant with a Tn*5* transposon insertion in the *lysR1* gene. The *E. amylovora lysR1* gene is predicted to encode the DNA-binding transcriptional regulator LysR1, which is the eponymous founder of the LysR-type family of transcriptional regulators (LTTRs) first described in *Escherichia coli* (Stragier and Patte 1983). *E. coli lysR* mutants are auxotrophic for the amino acid lysine, and *E. coli* LysR regulates expression of the *lysA* lysine biosynthesis gene (Stragier et al. 1983). We previously discovered that lysine auxotrophy leads to a severe defect in *E. amylovora* virulence through the characterisation of a *lysA* mutant (Klee, Sinn, and Finley 2019). We therefore thought it was possible that the *E. amylovora lysR1* mutant had decreased virulence due to a reduced ability to synthesise lysine.

However, members of the LTTR family have been implicated in the regulation of plant pathogen virulence in various ways in other pathosystems, in both positive and negative regulatory modes. For example, in the soft rot pathogen *Dickeya dadantii*, mutation of the *hdfR* gene and another LTTR-encoding gene (Locus Dda3937_04075) reduces fitness in chicory leaves as determined using a transposon sequencing approach (Royet et al. 2019). In *Agrobacterium tumefaciens*, a *vtlR* deletion mutant shows reduced tumour formation in potato (Eisfeld et al. 2021). On the other hand, mutation of the *Ralstonia solanacearum crgA* gene results in increased swimming motility and more rapid wilt disease development on tomato plants following soil drench inoculation, indicating that CrgA is a negative regulator of motility in this pathogen (Fan et al. 2020). Mutation of the *D. dadantii* (*Erwinia chrysanthemi*) LTTR gene *pecT* results in increased tissue maceration in chicory and celery and increased production of pectate lyase enzymes (Castillo and Reverchon 1997). Similar results were obtained with a *Pectobacterium carotovorum* (*Erwinia carotovora*) *hexA* mutant, which has increased virulence in potato tubers (Harris et al. 1998).

The varied effects of LTTR mutants on virulence activities in other bacterial genera raised the question of whether *E. amylovora* LysR1 influences *E. amylovora* virulence in some way other than, or in addition to, simply affecting lysine biosynthesis. We therefore undertook a detailed genetic, physiological and molecular analysis of the influence of the *lysR1* gene on the ability of *E. amylovora* to induce fire blight disease development.

2 | Materials and Methods

2.1 | Bacterial Strains and Culture

A spontaneous nalidixic-acid derivative of Pennsylvania isolate HKN06P1 (6P1 nal^R; Lee et al. 2010; Klee et al. 2022) served as wild-type (WT) *E. amylovora* in this study. *E. amylovora* and *E. coli* were routinely grown in lysogeny broth (LB) at 28°C and 37°C, respectively. M9 minimal medium (M9) and amylovoran minimal medium (AMM) are described by Klee et al. (2022). Media were supplemented with the following as needed: 15 g/L agar, 20 μg/mL nalidixic acid (nal), 50 μg/mL kanamycin (kan), 100 μg/mL carbenicillin (carb), 25 μg/mL chloramphenicol (cam), 25 μg/mL streptomycin (strep) and 50 μg/mL spectinomycin (spec). All bacterial strains employed in this study are described in Table S1.

2.2 | Mutant Generation and Complementation

Transposon mutations were created and identified using the EZ-Tn5 < R6Kγori/KAN-2 > kit (Illumina) as described previously (Klee et al. 2022). The *lysR1* deletion from 6P1 nal^R was made via allelic exchange (Datsenko and Wanner 2000). Deletion primers were used in a PCR with pKD3 as the template to generate a chloramphenicol resistance (*cam^R*) cassette flanked by DNA homologous to regions upstream and downstream of *lysR1*. The PCR product was electroporated into WT *E. amylovora* carrying pKD46 to allow allelic exchange, and resultant colonies were selected on LB with nal, cam and carb. The deletion was confirmed by colony PCR with *lysR1*- and *cam^R*-specific primers.

Complementation primers were designed to allow expression of WT *lysR1* and *lysA* under their native promoters. Primer pairs encompass the coding region of each gene and the shared intergenic region between the divergently transcribed genes and are flanked by restriction sites to allow subcloning (Table S1).

The TOPO-TA cloning kit (Invitrogen) was used to ligate WT *lysA* and *lysR1* PCR products into cloning vector pCR2.1 for amplification and verification by DNA sequencing. DNA sequencing was performed at the Huck Institute Genomics Core Facility at Penn State. The products were excised from pCR2.1 by digestion with appropriate restriction enzymes and ligated into similarly cut low-copy vector pCE (Klee et al. 2022). Integration into pCE was confirmed by restriction mapping. Plasmids were introduced into target strains by electroporation and confirmed by growth on selective media and colony PCR. Table S1 lists all plasmids and primers used in this study.

2.3 | Plant Assays

The ability of strains to induce fire blight symptoms in immature apple fruitlets was assayed as described previously (Klee et al. 2019). Briefly, fruitlets were wounded and inoculated with 2×10^6 colony-forming units (cfu) of bacteria. Six days after inoculation (dai), photographs of apples were taken. At 8 dai, disease severity was assessed using the rating system of Klee, Sinn, and McNellis (2019) and expressed as the ratio of the severity induced by mutant strains to the severity induced by WT. Four (for photographs) or 10 (for disease severity) fruitlets per strain were inoculated in each experiment, and each type of experiment was conducted at least three times. Within each disease severity experiment, statistically significant differences between mutant strains and the WT were determined by Student's *t* tests ($p \leq 0.01$).

Virulence in apple seedlings was tested on potted, second leaf (2 years after grafting), greenhouse grown cv. Gala trees with Malling 9 (M.9) rootstocks. Inoculation was by shoot tip wounding as detailed by Klee et al. (2020). Disease severity was expressed as the length of blighted shoot relative to the entire shoot length. Five shoots per tree were inoculated on five trees for each bacterial strain for a total of 25 shoots inoculated per strain. Trees were arranged in a randomised complete block. Symptom development was assessed weekly for 3 weeks after inoculation. The entire experiment was performed twice. Data were analysed by ANOVA followed by Tukey's HSD ($p \leq 0.01$).

Hypersensitive responses (HR) were tested by infiltrating the abaxial surfaces of leaves of 5- to 6-week-old tobacco plants (*Nicotiana tabacum* 'Glurk') with $OD_{600nm} = 0.1$ (10^8 cfu/mL) suspensions of *E. amylovora* strains in 10 mM $MgCl_2$. Photographs were taken 48 h after infiltration. The experiment was performed twice using four to six plants per experiment.

2.4 | Growth in Minimal Medium

Bacterial strains were grown overnight in LB, then washed twice with 10 mM $MgCl_2$ and suspended to $OD_{600nm} = 0.05$ in M9 medium with or without 10 mM lysine. Samples were loaded into clear 96-well plates, with six replicate wells per sample type and their corresponding blanks (medium without bacteria), and plates were sealed with Breathe-Easy film (Diversified Biotech). Cultures were grown at 28°C with gentle agitation, and OD_{600nm} was measured at 24 and 48 h with a SpectraMax i3x (Molecular Devices).

2.5 | Virulence Factor Assays

For the amylovoran and levansucrase assays, bacteria were grown in AMM for 20 h or in LB overnight, respectively. The amylovoran assays were conducted as described by Klee et al. (2020). Amylovoran production was normalised to bacterial cell number as determined by serial dilution plating as previously described (Klee et al. 2020). Levansucrase activity was determined by the method of Zhang and Geider (1999) as detailed by Lee et al. (2010). In each assay, there were three biological replicates per strain, and the experiments were performed at least three times with similar results each time.

Swimming motility was assayed as previously described (Klee et al. 2018). Briefly, bacteria were grown in LB overnight, then diluted 1:50 in fresh LB and grown for 4 h before washing with $MgCl_2$ and adjusting to ~10^6 cfu/mL in 10 mM $MgCl_2$. A 5 μL droplet of bacterial suspension was dotted onto 0.3% LB agar plates and the cultures were incubated at 28°C for 36 h, after which plates were photographed, and average cross-section diameters of swimming areas were measured using NIH ImageJ software. Swimming areas (cm^2) were calculated based on the average cross-section diameters and assuming a circular shape ($A = \pi r^2$). Four or five agar plates were assessed in each of three replicate experiments. For amylovoran, levansucrase, and motility assays, pairwise Student's *t* tests were used to determine significant differences between mutants and WT within a replicate experiment ($p \leq 0.01$). Lipopolysaccharide (LPS) analysis was performed on equal numbers of cells (as determined by OD_{600nm} measurement) from overnight LB cultures as previously described (Klee et al. 2020).

2.6 | RNA Isolation and Sequencing

RNA purification from WT and *lysR1*-Tn*5*, and subsequent RNA sequencing (RNA-Seq) analyses were performed as previously described (Klee et al. 2022). In brief, total RNA was extracted from each of three independent WT and *lysR1*-Tn*5* cultures grown in AMM, the rRNA was depleted, and single-end, 75 bp RNA-Seq was carried out on an Illumina NextSeq 550 instrument at the Penn State Genomics Core Facility. Expression data were obtained for 3446 of the 3706 predicted *E. amylovora* chromosomal coding regions (Smits et al. 2010). The data were analysed using Geneious Prime and the DESeq2 method (Love et al. 2014).

3 | Results

3.1 | *lysR1* and *lysA* Mutant Virulence Phenotypes

During ongoing screening of *E. amylovora* Tn*5* transposon insertion mutants for reduced virulence using an immature apple fruitlet fire blight model system (Klee, Sinn, and McNellis 2019), we identified a mutant with a Tn*5* transposon insertion in the *lysR1* gene (*lysR1*-Tn*5*; Figure 1). Subsequently, a *lysR1* allelic exchange mutant was created (Δ*lysR1*; Figure 1), wherein the entire 921 nucleotide coding region of the *lysR1* gene was replaced with a chloramphenicol resistance cassette via λRed recombination (Datsenko and Wanner 2000). The predicted protein encoded by *E. amylovora lysR1* has 75.9% amino acid identity and 87.5% amino acid similarity with the *LysR* protein first identified in *E. coli* (Stragier and Patte 1983).

Members of the LTTR protein family are frequently located adjacent to, but transcribed in a divergent direction from, their target genes, although the genes can be further apart (Maddocks and Oyston 2008). The *E. amylovora lysR1* gene is immediately adjacent to its presumed regulatory target gene *lysA* and is transcribed in the opposite direction (Figure 1). The *lysR* and *lysA* genes are similarly arranged in *E. coli* (Stragier and Patte 1983). The *E. amylovora lysA* gene

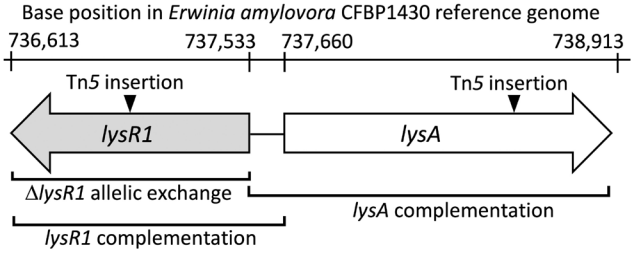

FIGURE 1 | Genomic context of the *Erwinia amylovora lysR1* and *lysA* genes, with locations of Tn*5* transposon insertions, the Δ*lysR1* allelic exchange region and regions used for genetic complementation indicated.

is predicted to encode the enzyme diaminopimelate decarboxylase, which catalyses the final step in the biosynthesis of L-lysine by the decarboxylation of *meso*-1,6-diaminopimelate in most prokaryotes (Velasco et al. 2002). We previously described a single Tn*5* transposon insertion mutant of *E. amylovora lysA* that was auxotrophic for lysine and had reduced pathogenicity on apple fruitlets (Klee, Sinn, and Finley 2019). Additional characterisation of this *E. amylovora lysA* mutant is presented herein.

The *lysR1*-Tn*5* and Δ*lysR1* mutants both produced less of the diagnostic fire blight symptoms of bacterial ooze and tissue necrosis compared to the WT when inoculated onto immature apple fruitlets (Figure 2). A plasmid-borne copy of *lysR1* (p*lysR*; Table S1 and Figure 1) restored virulence to the corresponding mutants, whereas the empty vector (pEV) control did not (Figure 2). Apple fruitlet symptom development was quantified using a disease rating scale (Klee et al. 2019) and expressed as a disease severity ratio relative to symptoms caused by WT *E. amylovora*, as described previously (Klee et al. 2019). A severity ratio of 1 represents the WT level of disease, and ratios below 1 represent less than WT disease severity. Disease severity ratios of the *lysR1*-Tn*5* and Δ*lysR1* mutants were significantly lower than WT, and the p*lysR1* plasmid restored the disease severity ratios of the mutants to the WT level (Figure 3). These results confirm that *lysR1* is required for full virulence of *E. amylovora* on apple fruitlets.

In addition, the *lysR1*-Tn*5* mutant produced dramatically attenuated symptoms of shoot blight necrosis compared to the WT over the course of 3 weeks on apple trees inoculated in a greenhouse (Figure 4). The p*lysR* plasmid complemented the virulence of the *lysR1*-Tn*5* mutant on apple shoots, although at the 1- and 2-week time points the necrosis caused by the complementation strain was less extensive than that caused by the WT (Figure 4). By 3 weeks after inoculation, both the complementation strain and

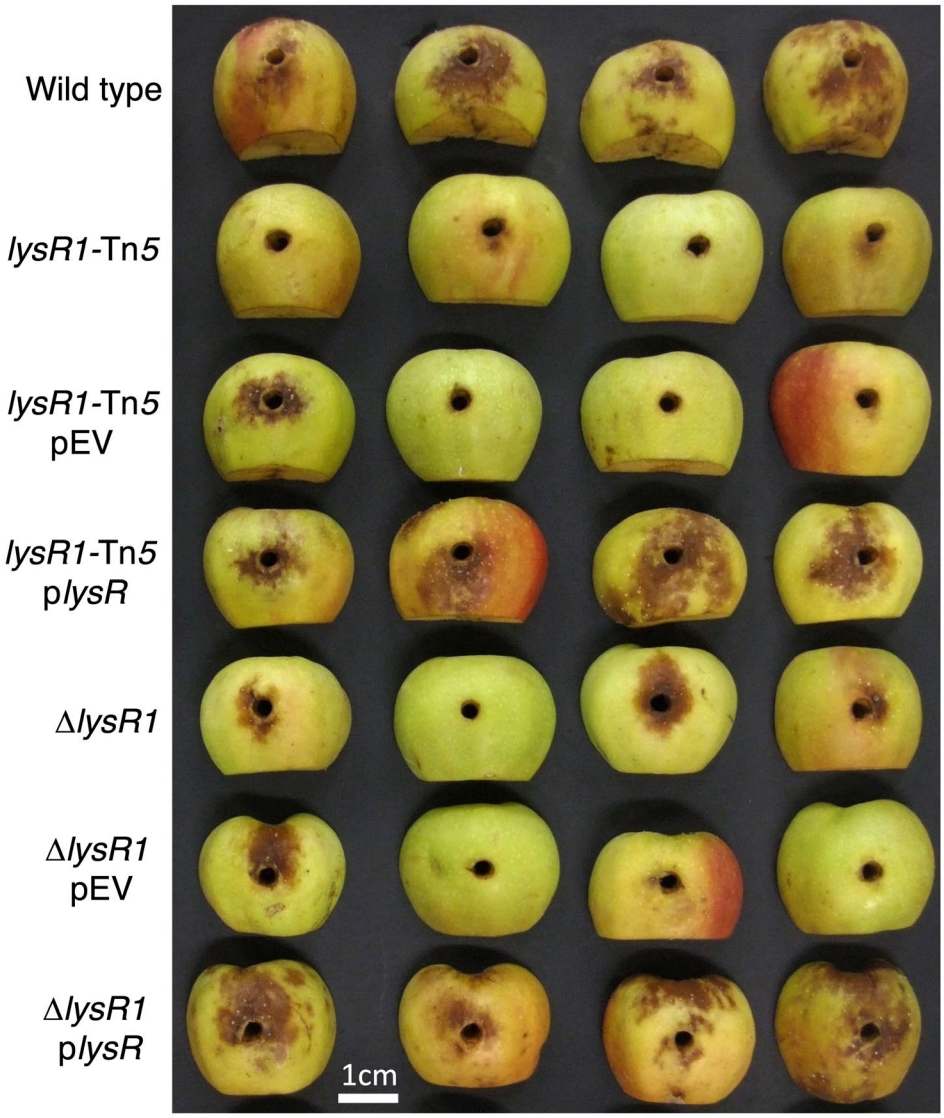

FIGURE 2 | Visual virulence phenotypes and genetic complementation of *Erwinia amylovora lysR1* mutants in apple fruitlets. Gala apple fruitlets were photographed at 6 days after inoculation with 2×10^6 cfu of the indicated *E. amylovora* strains. Fire blight symptoms include necrosis and bacterial ooze. The experiment was performed at least three times with similar results each time; results of a representative experiment are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

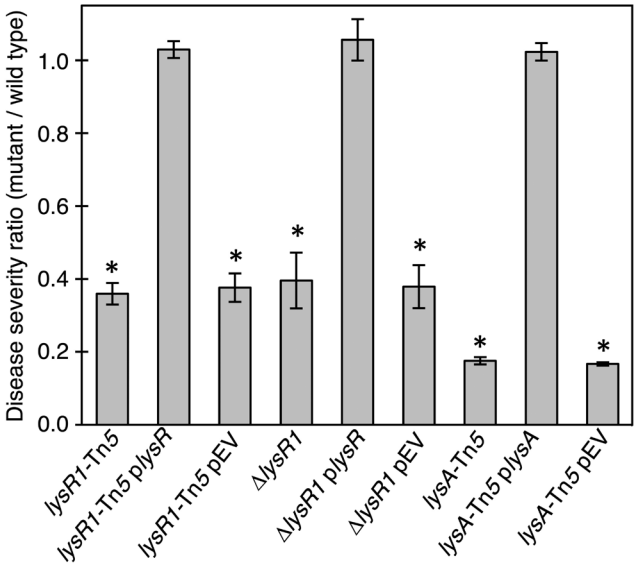

FIGURE 3 | Quantitative virulence phenotypes and genetic complementation of *Erwinia amylovora lysR1* and *lysA* mutants in apple fruitlets. Disease severity in Gala apple fruitlets ($n = 10$ fruitlets inoculated per bacterial strain) was assessed at 8 days after inoculation with 2×10^6 cfu of the indicated *E. amylovora* strains using a disease severity scale (Klee et al. 2019) and expressed as a disease severity ratio of mutant to wild type (Klee et al. 2019). Error bars indicate standard error. Asterisks indicate a statistically significant difference from the wild type using Student's *t* test ($p < 0.01$); $n = 10$ fruitlets per strain. The experiment was performed at least three times with similar results each time; results of a representative experiment are shown.

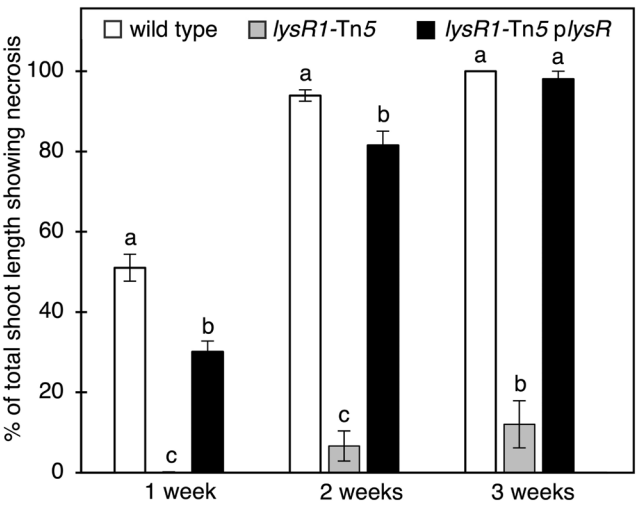

FIGURE 4 | Disease severity on shoots of greenhouse-grown Gala apple trees wound-inoculated with the indicated *Erwinia amylovora* strains at 1, 2 and 3 weeks post-inoculation. Error bars indicate standard error. Within each time point, bars with different letters have a statistically significant difference as determined by analysis of variance (ANOVA) with post hoc Tukey tests; $p \leq 0.01$; $n = 25$ shoots per strain. The experiment was performed twice with similar results both times; the results of a representative experiment are shown.

the WT caused nearly complete necrosis of inoculated shoots. These results indicate that *lysR1* is required for full virulence of *E. amylovora* in apple tree shoots.

The single *lysA*-Tn*5* mutant we described previously was part of an extensive survey of auxotrophic *E. amylovora* mutants and had not been confirmed by genetic complementation (Klee et al. 2019). Here, a plasmid-borne copy of *lysA* (p*lysA*; Table S1; Figure 1) was used to complement the virulence phenotype of the *lysA*-Tn*5* mutant in apple fruitlets (Figures 3 and 5), confirming that *lysA* is required for full virulence of *E. amylovora* on apple fruitlets.

3.2 | *lysR1* and *lysA* Mutant Growth in Minimal Medium

lysA and *lysR* mutants were both reported as being auxotrophic for lysine in *E. coli* (Stragier et al. 1983). The *E. amylovora* *lysR1*-Tn*5*, Δ*lysR1* and *lysA*-Tn*5* mutants were tested for lysine auxotrophy by growth in liquid minimal medium with and without lysine supplementation. As expected, the *lysA*-Tn*5* mutant could not grow in minimal medium without lysine supplementation (Figure 6), consistent with our earlier study (Klee et al. 2019). Unexpectedly, however, the *lysR1*-Tn*5* and Δ*lysR1* mutants grew as well as the WT in minimal medium without lysine supplementation (Figure 6). Thus, mutation or deletion of *lysR1* in *E. amylovora* does not result in lysine auxotrophy.

3.3 | *lysR1* Mutant Virulence Factor Expression

These results indicate that while the virulence defect of the *E. amylovora* *lysA*-Tn*5* mutant may be attributable to lysine auxotrophy, this is not the case for *lysR1* mutants. We therefore assessed the expression of major known *E. amylovora* virulence factors in the mutants to explore potential causes for the reduced virulence of *lysR1* mutants.

The exopolysaccharide (EPS) amylovoran is an essential virulence factor for *E. amylovora* (Bellemann and Geider 1992; Bugert and Geider 1995), and reduced levels of amylovoran are associated with reductions in virulence (Klee et al. 2020, 2022; Koczan et al. 2009; Lee et al. 2010). Amylovoran production was significantly reduced in both *lysR1*-Tn*5* and Δ*lysR1* compared to the WT (Figure 7). Amylovoran production was restored to levels matching or exceeding the WT by complementation plasmid p*lysR*, while the empty vector pEV plasmid did not have this effect (Figure 7). The *lysA*-Tn*5* mutant was not included in this experiment because it failed to grow in AMM, the minimal medium used to induce amylovoran production by *E. amylovora*.

The *E. amylovora* type III protein secretion system (T3SS) is required both for virulence in host plants and for elicitation of the hypersensitive cell death defence reaction (HR) in fire blight non-host tobacco (*N. tabacum*) plants (Kim et al. 1997). When infiltrated into tobacco leaves, the WT and mutants *lysR1*-Tn*5*, Δ*lysR1* and *lysA*-Tn*5* all induced the development of tissue necrosis in the infiltrated areas, while a T3SS mutant (*hrcC*-Tn*5*) failed to elicit a response (Figure 8). This indicates that the mutants all retain the ability to trigger the HR in tobacco and that the T3SS can operate normally in the *E. amylovora* *lysR1* and *lysA* mutants.

FIGURE 5 | Visual virulence phenotypes and genetic complementation of an *Erwinia amylovora lysA* mutant in apple fruitlets. Gala apple fruitlets were photographed at 6 days after inoculation with 2×10^6 cfu of the indicated *E. amylovora* strains. The experiment was performed three times with similar results each time; the results of a representative experiment are shown. Fire blight symptoms include necrosis and bacterial ooze. [Colour figure can be viewed at wileyonlinelibrary.com]

Lipopolysaccharides (LPS) embedded in the outer leaflet of the outer membrane serve as a physical barrier for bacterial cells (Rosenfeld and Shai 2006) and play a role in biofilm formation (Beveridge et al. 1997). LPS contributes to the virulence of *E. amylovora* (Ray et al. 1986; Berry et al. 2009; Klee et al. 2020) and other gram-negative pathogenic microbes such as *Salmonella enterica* (Thomsen et al. 2003). When analysed by silver-stained polyacrylamide gel electrophoresis (Hitchcock and Brown 1983), LPS banding patterns in extracts from negative control *E. amylovora* mutant strain *rfbA1*-Tn*5* (Klee et al. 2020) showed a virtual absence of LPS banding compared to the WT (Figure 9). In contrast, LPS banding profiles in the *lysR1*-Tn*5*, Δ*lysR1* and *lysA*-Tn*5* mutants all appeared like the WT (Figure 9). Thus, the *lysR1* and *lysA* mutants do not appear to have a disruption in LPS production.

We also assessed swimming motility and levansucrase enzyme activity in the *lysR1*-Tn*5* mutant, although these are not expected to impact virulence in the apple fruitlet or tree shoot inoculation virulence tests used in this study. No significant differences between *lysR1* mutants and the WT were noted in swimming motility (Figure S1) or levansucrase activity (Figure S2). Swimming plays a role in flower infection (Bayot and Ries 1986), and levansucrase enzyme produces the neutral EPS levan in many but not all *E. amylovora* strains (Bereswill et al. 1997). In summary, only amylovoran production was affected in *E. amylovora lysR1* mutants among all the virulence-related phenotypes examined.

3.4 | RNA Sequencing

To better understand the *E. amylovora lysR1*-Tn*5* mutant phenotype, global gene expression in the *lysR1*-Tn*5* mutant was compared to that in the WT using RNA-Seq as described previously (Klee et al. 2022). The expression data of the biological replicates showed a clear separation based on genotype when plotted via principal component analysis, indicating that the *lysR1*-Tn*5* mutant had a transcriptional profile distinct from that of the WT (Figure S3a). Differentially expressed genes were defined as having a $\log_2$ fold-change (LFC) of >1 or <1 and an adjusted p value <0.01. Overall, the effect of mutation *lysR1* on global *E. amylovora* gene expression was modest, with only 34 genes meeting the LFC cut-off criteria (Figure S3b). The LFCs of the differentially expressed genes had a modest range, from a high of 1.799 (*ftnA*) to a low of −1.808 (*ssuE*). All genes meeting the LFC cut-off criteria had adjusted p values well below the 0.01 cut-off (Table S2). Of the differentially expressed genes, 27 were reduced in expression in *lysR1*-Tn*5* compared to the WT, whereas seven were increased. The differential expression data are summarised in Table S2 and File S1 contains the complete data.

Expression of *lysA* was reduced in the *lysR1*-Tn*5* mutant compared to the WT, with an LFC of −1.525. Notably, the amylovoran biosynthesis operon *ams* genes (Bugert and Geider 1995) were not differentially expressed in the *lysR1*-Tn*5* mutant compared to the WT (File S1). Furthermore, the differentially expressed genes did not include any known *E. amylovora* virulence systems or components (Table S2). Despite the presence of a Tn*5* insertion, areas of *lysR1* were still transcribed in the *lysR1*-Tn*5* mutant (Figure S4), producing an LFC of 1.248 (Table S2).

4 | Discussion

The present study demonstrates that the *lysR1* gene of *E. amylovora* is essential for full virulence of this pathogen on apples. We

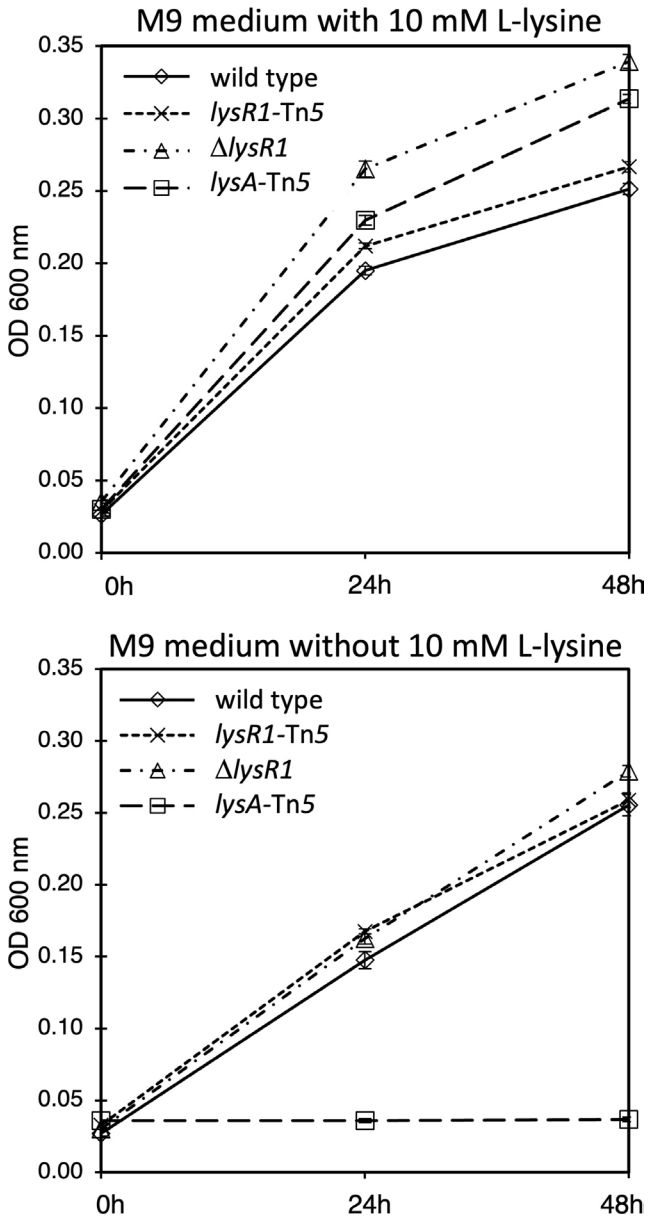

FIGURE 6 | Growth of the indicated *Erwinia amylovora* strains in shaken liquid M9 minimal medium culture with sorbitol as the carbon source, with and without 10 mM L-lysine supplementation, measured spectrophotometrically as optical density (OD) at 600 nm. Bars indicate standard error ($n = 6$ cultures per strain). The experiment was performed three times with similar results each time; the results of a representative experiment are shown.

conclude that the reduced virulence of *E. amylovora lysR1* mutants is not due to lysine auxotrophy, because the *lysR1* mutants were, unexpectedly, prototrophic. Rather, a significant reduction in amylovoran production is likely to be the major contributor to the attenuated virulence of *E. amylovora lysR1* mutants. However, we conclude that the reduction in amylovoran production in *lysR1* mutants is an indirect effect of loss of LysR1 rather than any direct regulatory role of LysR1 in *ams* gene expression because no differences in *ams* gene expression were detected in the *lysR1*-Tn*5* mutant compared to the WT.

We also conclude that LysR1 probably contributes to the regulation of *lysA* gene expression, as expected based on results in

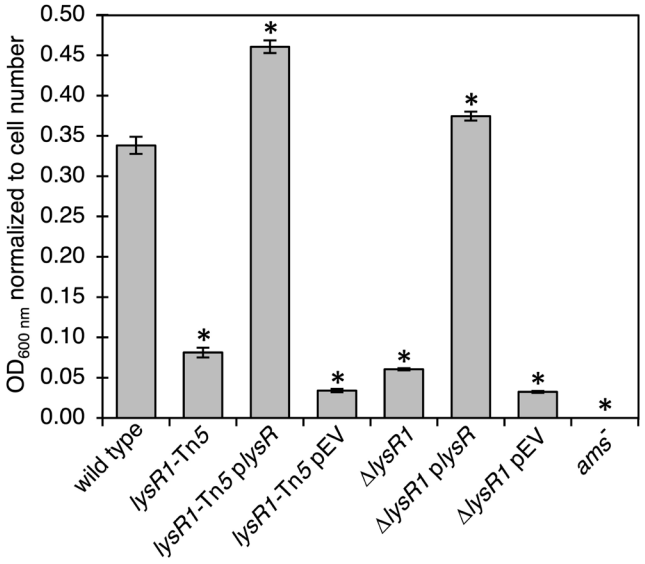

FIGURE 7 | Amylovoran exopolysaccharide production by the indicated *Erwinia amylovora* strains grown in amylovoran minimal medium (AMM), normalised to cell number. Error bars indicate standard errors and asterisks indicate a statistically significant difference from the wild type using Student's *t* test ($p \leq 0.05$) $n = 3$ per strain. The *ams*⁻ mutant carries a Tn*5* transposon insertion in the *amylovoran synthesis* (*ams*) operon promoter region (Klee et al. 2022; Table S1) and serves as a negative control for amylovoran synthesis. The *lysA*-Tn*5* mutant was not included in this experiment because it cannot multiply in AMM, which is a minimal medium. The experiment was performed three times with similar results each time; results of a representative experiment are shown. $OD_{600\ nm}$, optical density at 600 nm.

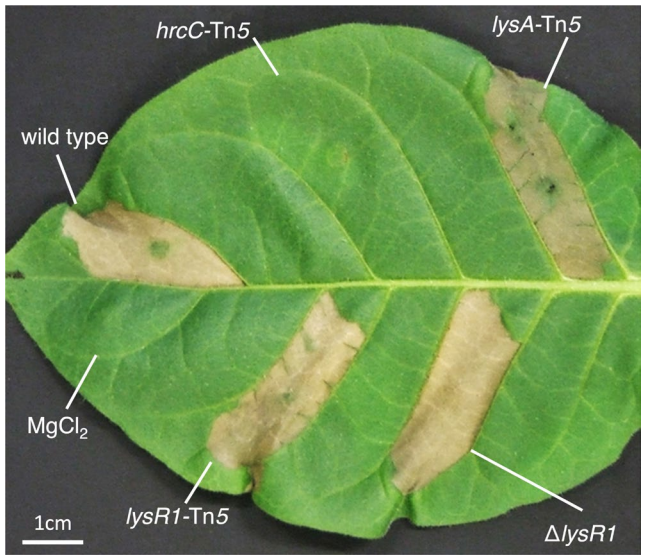

FIGURE 8 | Elicitation of the hypersensitive reaction in tobacco (*Nicotiana tabacum* 'Glurk') leaves after infiltration with a suspension of 10^8 cfu/mL of the indicated *Erwinia amylovora* strains or 10 mM magnesium chloride ($MgCl_2$). Strain *hrcC*-Tn*5* is a negative control with a defective type III secretion system (Klee, Sinn, and Finley 2019; Table S1). Collapse was noted within 24 h after infiltration; photograph was taken at 48 h after infiltration for improved contrast. Four to six different plants were used in each experiment, and the experiment was performed twice with similar results each time. A representative leaf is shown. [Colour figure can be viewed at wileyonlinelibrary.com]

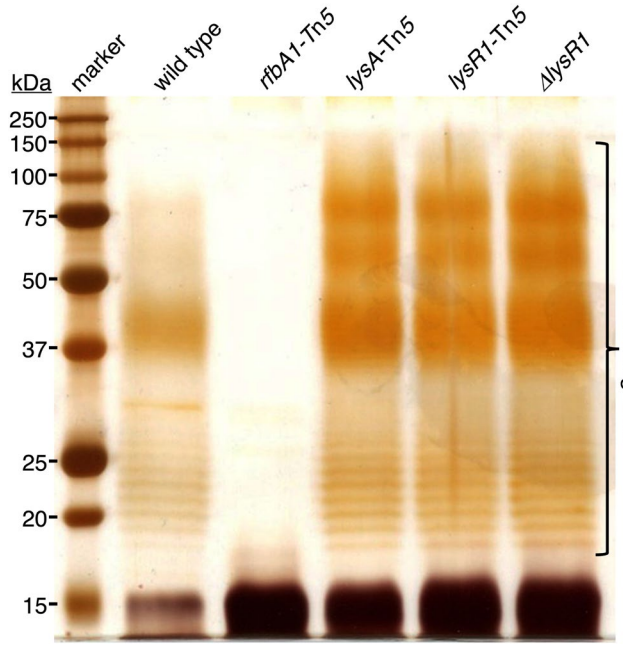

FIGURE 9 | Lipopolysaccharide (LPS) production by the indicated *Erwinia amylovora* strains grown in lysogeny broth as visualised by polyacrylamide gel electrophoresis followed by silver staining. *rfbA1*-Tn*5* is a negative control *E. amylovora* mutant unable to produce wild-type LPS (Klee et al. 2020; Table S1). Marker, protein molecular weight marker lane with sizes indicated in kDa. The experiment was conducted three times with the same result; results of a representative experiment are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

E. coli (Stragier et al. 1983). However, the reduction in *lysA* expression was insufficient to result in lysine auxotrophy in the *lysR1*-Tn*5* mutant. Therefore, we conclude that *lysA* can be expressed sufficiently in the absence of LysR1 in *E. amylovora*. This would account for the prototrophy of the *E. amylovora lysR1* mutants, which is a sharp contrast with the lysine auxotrophy of *E. coli lysR1* mutants (Stragier et al. 1983).

Although reads were obtained mapping to *lysR1* in the *lysR1*-Tn*5* mutant in the RNA-Seq experiment, these are unlikely to result in the production of any functional LysR1 protein. The phenotypes of the *lysR1*-Tn*5* and Δ*lysR1* mutants were identical in all assays where they were tested together, which included the fruitlet disease assays and all the major virulence factor tests. The identical phenotypes of the *lysR1*-Tn*5* and Δ*lysR1* mutants indicate that the transposon insertion in *lysR1*-Tn*5* completely knocked out LysR1 function.

The effect of mutation of *lysR1* in *E. amylovora* was modest in terms of the number of genes affected and the magnitude of their expression differences. In contrast, an RNA-Seq study of the *E. amylovora* ppGpp stringent response regulator mutant ppGpp^0 detected 1314 differentially expressed genes compared to WT in a minimal medium, with LFCs ranging as widely as ±6 or greater (Yang et al. 2020). Similarly, in *A. tumefaciens*, deletion of LTTR family member *vtlR* altered the expression of over 1200 genes (Eisfeld et al. 2021). Despite the modest effects on global gene expression due to mutation of *lysR1*, the negative effect of *lysR1* mutation on *E. amylovora* virulence in apple was clear and significant.

As a member of the LTTR family, *E. amylovora* LysR1 is predicted to be a DNA-binding transcriptional regulatory protein. Many other DNA-binding transcriptional regulators contributing to virulence have been previously identified in *E. amylovora*. The HrpL alternative sigma factor controls expression of T3SS components and is required for *E. amylovora* virulence (McNally et al. 2012; Wei and Beer 1995). Interestingly, HrpL also negatively regulates flagellar motility, which contributes to *E. amylovora* infection of non-injured apple seedlings (Cesbron et al. 2006). The expression of *hrpL* is regulated by the alternative sigma factor 54 (σ^{54}) RpoN and the σ^{54} enhancer binding protein HrpS, both of which are required for fire blight disease development (Ancona et al. 2014; Lee et al. 2016). The RcsA and RcsB proteins together associate with the amylovoran biosynthesis operon promoter to regulate the production of amylovoran exopolysaccharide (Kelm et al. 1997). While not required for fire blight disease development in apple seedlings (Anderson et al. 1998) or pear plants (Santander et al. 2014), the alternative sigma factor RpoS positively contributes to swimming motility, amylovoran production, stress tolerance and disease instigation in immature loquat (*Eriobotrya japonica*) fruits (Santander et al. 2014). The two-component system (Beier and Gross 2006) HrpX/HrpY regulates *hrpL* expression (Wei et al. 2000) possibly via HrpS (Ancona et al. 2014; Lee and Zhao 2018). Interestingly, the leucine-responsive transcription factor Lrp is required for full virulence of *E. amylovora* on apple tree shoots and full expression of several virulence factors (Schachterle and Sundin 2019), providing another link between amino acids and virulence.

While a defect in amylovoran biosynthesis probably contributes to the reduced virulence of *E. amylovora lysR1* mutants, the link between LysR1 and amylovoran production remains to be elucidated. While transcription factor regulon studies in *E. amylovora rpoN* (Yang et al. 2023) and *hrpL* (McNally et al. 2012) mutants yielded profiles consistent with regulation of virulence, this was not the case with the *lysR1*-Tn*5* mutant in the present study. Future studies could use site-directed mutagenesis to determine whether disruption of genes differentially expressed in *lysR1*-Tn*5* compared to the WT results in alterations in amylovoran production or reductions in virulence, potentially identifying additional genes directly or indirectly required for these processes (McNally et al. 2012).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Upon acceptance for publication, the RNA-Seq data presented will be deposited in a MIAME-compliant database for public access. Strains, plasmids and other materials described herein will be provided to researchers upon request to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.